

Microcin E492 Is an Unmodified Peptide Related in Structure to Colicin V

Anne-Marie Pons,¹ Nathalie Zorn,² David Vignon,¹ François Delalande,² Alain Van Dorsselaer,² and Gilles Cottenceau^{1*}

Laboratoire de Spectrométrie de Masse Bioorganique, Université Louis Pasteur, 67000 Strasbourg Cedex,² and Laboratoire de Génie Protéique et Cellulaire, Université de La Rochelle, 17042 La Rochelle Cedex 01,¹ France

Received 13 June 2001/Returned for modification 3 August 2001/Accepted 3 October 2001

The pore-forming microcin E492 was purified by solid-phase extraction and reversed-phase high-pressure liquid chromatography. Its molecular mass was 7,886 Da. The entire 84-amino-acid sequence was determined. There is no postranslational modification in the secreted microcin, and the sequence has homologies with the sequence of the microcin colicin V.

Microcin E492 (Mcc E492) is a hydrophobic antibacterial peptide that is acid and heat resistant and active against strains of *Escherichia coli*, *Klebsiella*, *Salmonella*, *Citrobacter*, *Enterobacter*, and *Erwinia* (1). Its structural gene (*mceA*) was cloned (9) and sequenced (5). *MceA* codes for a precursor of 99 or 103 amino acids that is cleaved at either amino acid 15 or amino acid 19.

Its antibacterial activity is achieved through membrane depolarization (2), induced by the formation of pores in the membrane (4). The only other described microcin that inhibits cellular growth by disrupting membrane potential is microcin colicin V (Col V) (10), but until now, it has not been possible to demonstrate that it is a channel-forming microcin.

Thus, so far Mcc E492 is the only pore-forming microcin that has been described and represents a peptide model for a novel class of low-molecular-weight, pore-forming bacteriocins. So, to understand its biochemical properties, processing, and post-translational modifications, the amino acid sequence of Mcc E492 was determined.

Bacterial strains, culture conditions, and microcin detection. Mcc E492 producer was a recombinant *E. coli* strain, strain VCS 257 (Amp^r). The indicator strain for antimicrobial activity was *E. coli* DH5 α . Preparation of medium M63, preservation of strains, and culture conditions were as described by Sable et al. (7). Microcin activities were determined as described by Portrait et al. (6).

Purification of the microcin. Mcc E492 was prepurified by solid-phase extraction on Sep-Pak Plus C₁₈ cartridges (Waters, Saint Quentin en Yvelines, France) from a heated (10 min, 120°C) and centrifuged culture supernatant of an Mcc E492 producer in medium M63 (24 h, 37°C). The solid phase, loaded with 400 ml of supernatant, was washed with 20 ml of distilled water and then with 20 ml of a 50%-50% (vol/vol) water-methanol mixture. Mcc E492 was eluted with 30 ml of a 65%-35% (vol/vol) water-acetonitrile mixture and was then purified by reversed-phase high-pressure liquid chromatography (RP-

HPLC) with a C₁₈ column (300 Å pore size, 15 μ m granulometry, 300 by 3.9 mm [inner diameter]; Delta-Pak; Waters). The mobile phase (flow rate, 0.6 ml min⁻¹) was water with 0.1% trifluoroacetic acid (TFA) (eluent A) and acetonitrile with 0.1% TFA (eluent B). The gradient was 30% eluent B for 10 min, 30 to 50% eluent B in 2 min, and 50 to 63% eluent B in 13 min. The optical density (OD) was measured at 215 nm. Mcc E492 appeared as a single sharp peak (retention time, 20.5 min). The Mcc E492 fractions were pooled, freeze-dried, and stored at 4°C.

Analysis of the microcin. The molecular mass of the microcin was determined on a Biflex matrix-assisted laser desorption ionization–time of flight–mass spectrometer (MALDI-TOF-MS; Brüker, Bremen, Germany) in the linear mode. Ionization was accomplished with the 337 nm beam from a nitrogen laser with a 3-Hz repetition rate. Spectra were obtained in the positive mode and were calibrated with the single and double charged peaks of horse heart myoglobin (2 pmol μ l⁻¹) at *m/z* 16,952.5 and 8,476.8, respectively. A sample for analysis was prepared by the sandwich method, as described by Strub et al. (8).

Electrospray ionization mass spectrometry (ESMS) analysis was carried out on a VG BioQ mass spectrometer (Bio Tech, Manchester, United Kingdom). The sample diluted in 50:50 (vol/vol) water-acetonitrile with 1% formic acid solution was introduced into the ionization chamber through a capillary, and a 3.2-kV potential was applied between the end of the capillary and the first electrode. The solvent was evaporated by a nitrogen flow at 80°C. The calibration was performed with horse heart myoglobin (2 pmol μ l⁻¹), and the scanning ranged from *m/z* 500 to 1,500.

MALDI-TOF-MS analysis revealed a single peak with a molecular mass of 7,887.8 Da. By ESMS, the accurate molecular mass of Mcc E492 was determined to be 7,886.68 \pm 0.52 Da. Following retrieval of the mass spectrometry measurements, the peptide was subjected to Edman degradation. We obtained the first 59 NH₂-terminal residues.

Mcc E492 in 40 μ l of phosphate buffer (50 mM; pH 7.5) and 10 μ l of acetonitrile was digested with endoproteinase Asp-N (2 μ g, 4 h, 37°C). The resulting peptides were analyzed by liquid chromatography (LC)-mass spectrometry (MS) (Table 1). LC was separated by RP-HPLC (300 Å pore size, 5 μ m

* Corresponding author. Mailing address: LGPC, Pôle Sciences, Université de La Rochelle, Avenue Michel Crépeau, 17042 La Rochelle, France. Phone: 33-546-458-228. Fax: 33-546-458-247. E-mail: gilles.cottenceau@univ-lr.fr.

TABLE 1. Results of digestion of Mcc E492 with endoproteinase Asp-N

| Peptide (amino acid nos.) obtained by cleavage ^a | Expected mass (Da) | Mass (Da) determined by ESMS |
|--|-----------------------|---------------------------------|
| 50-84 | 3,342.6 | 3,343.06 ± 0.14 |
| 1-11 | 1,201.3 | 1,201.67 ± 0.17 |
| 12-49 | 3,378.8 | 3,378.56 ± 0.01 |
| 12-84 | 6,703.3 | 6,703.75 ± 0.16 |

^a Cleavage was obtained by digestion with endoproteinase Asp-N. The sequence from amino acids 50 to 77 was obtained by Edman degradation; the final sequence, SSS₈₀GSGS, was assumed from the nucleotide sequence of *mceA* (5).

granulometry, 125 by 2.1 mm [inner diameter], Nucleosil C₁₈ column; Macherey-Nagel, Düren, Germany). The mobile phase (flow rate, 0.25 ml min⁻¹) was water with 0.1% TFA (eluent A) and acetonitrile with 0.08% TFA (eluent B'). The gradient was 2% eluent B' for 10 min, 2 to 60% eluent B' in 60 min, and 60 to 80% eluent B' in 5 min, followed by isocratic elution with 80% eluent B'. The OD was measured at 215 nm. A T-shaped column divided the column effluent between ESMS and the detector with a split of 1/10 by volume. The ESMS was scanned over a mass range of *m/z* 300 to 1,700 at 7 s/scan. N-terminal sequencing of some of the peptides obtained was performed on a pulsed liquid automatic microsequencer (Procise cLC; Applied Biosystems Inc., Foster City, Calif.). Mcc E492 was found to be an 84-amino-acid polypeptide (predicted molecular mass, 7,886.57 Da). Its sequence is GETDPNTQLL₁₀NDLGNNMAWG₂₀AALGAPG *G *L⁺G₃₀*SA*A*LG*AA*G*G*A₄₀*L⁺QTVGQGLID₅₀ HG PVNVPIPV₆₀ LIGPSWNGSG₇₀SGYNSATSSS₈₀GSGS. The asterisks indicate conserved amino acids, and the plus signs indicate semi-conserved amino acids.

C-terminal sequencing by enzymatic digestion with carboxypeptidase P did not give a result, in accordance with the C-terminal sequence SSSGSGS. This is in agreement with the results of Lagos et al. (5) and confirms that the translation product of *mceA* is a precursor peptide cleaved at either amino acid 15 or amino acid 19 (depending on the methionine used as the start codon), providing a processed active Mcc E492 of 84 residues. By comparison with the nucleotide sequence of *mceA*, it also shows that after the cleavage of the leader sequence, Mcc E492 is not postranslationally modified.

The hydropathy profile of precursor peptide MceA (5) shows a single hydrophobic transmembrane domain that corresponds to amino acids 21 to 41 of Mcc E492. This sequence is probably responsible for the pore-forming activity of the microcin.

Comparison of the amino acid sequence Mcc E492 and the published partial amino acid sequence of Col V (3) shows that the fragment from amino acids 20 to 33 of Col V and the fragment from amino acids 27 to 41 of Mcc E492 share significant homologies, with 10 conserved and 2 semiconserved amino acids (Table 1). No protein sequences which had significant homology to Mcc E492 were identified from the EMBL or the Infobiogen data bank.

Nucleotide sequence accession number. The sequence of Mcc E492 is deposited in the SWISS-PROT protein sequence database (accession number P82962).

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